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Simultaneous determination of amylose content & unit chain distribution of amylopectins of cassava starches by fluorescent labeling/HPSEC

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Abstract

The fluorescent labeling of debranched starch/high-performance size-exclusion chromatography equipped with one column of Shodex OHpak SB-803HQ and two columns of SB-802.5HQ was proposed for simultaneous determination of amylose content and unit chain distribution of amylopectin of starch samples from four cassava varieties. Chromatograms obtained by refractive index (RI) response detection revealed that peaks of amylose and amylopectin fractions were completely separated, whereas only a peak of amylopectin was observed by fluorescent response detection. The amylose contents of cassava starch samples calculated from the RI response chromatograms were comparable to those determined by amperometric titration method and by overlaying the amylopectin profile on the profile of the starch isoamylolyzate. The values obtained for each starch sample were highly reproducible for duplicate analyses with standard deviations less than 0.6%. Distribution profiles of amylopectins from the starch isoamylolyzate were very similar to those obtained from fractionated amylopectins. Molar- and weight-based unit-chain fraction and ratio of A + B1/B2 + B3 were close to those derived from profiles of fractionated amylopectins. Differences of mole fraction of A, B1 and B2 + B3 chains and ratio of A + B1/B2 + B3 determined from starch and fractionated amylopectin chromatograms of all four cassava starches were less than 2.7, 2.7, 0.15 and 0.12%, respectively.

Keywords: Cassava starch; Amylose content; Labeling/HPSEC; Unit chain; Amylopectin

1. Introduction

Many of the physicochemical properties of starches that determine their suitability for particular end-uses are dependent on the ratio of amylose and amylopectin, as well as on their chain-length distributions. Amylose and amylopectin are quantitatively determined by several methods. The procedures based on different iodine binding capabilities of amylose and amylopectin, either spectrophotometric or amperometric titration, are perhaps the most widely utilized. Other alternative procedures, including differential scanning calorimetry, selective precipitation of amylopectin with concanavalin A, and chromatographic method either of native or of debranched starch, have also been developed to measure amylose content. Advantages and disadvantages of these procedures have been clearly stated by Batey and Curtin (1996).

High-performance size-exclusion chromatography (HPSEC) was considered to be promising for precise determination of amylose content. It was utilized for the fractionation and determination of amylose and amylopectin in an investigation by Kobayashi, Schwartz, and Lineback (1985). Instead of using carcinogenic solvents, Grant, Ostenson, and Rayas-Duarte (2002) used KOH and urea for solubilizing starch, and deionized distilled water as eluant. Sargeant (1982) reported a method of amylose determination which relied on debranching the starch by isoamylase and quantifying the amount of longer chain fraction (from amylose) and shorter chain fraction (from amylopectin) by size-exclusion chromatography (Sepharose CL-6B). Batey et al. (1996) quantitated the amylose content by separation of debranched starch with HPSEC on hydrophilic column (Ultrahydrogel 250), using a water-based mobile phase. The authors stated that starches of up to 30–35% amylose can be analyzed by this method; however, precipitation of the amylose may occur if the starches contain higher amylose content. They also suggested that this

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method may be a preferable method for measuring small differences in starches of similar amylose content, since it is more reproducible than the blue value method (varying by less than 0.5%).

HPSEC with a differential refractometer and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) have been frequently used for the examination of the chain-length distribution of amylopectin. These methods measure essentially a weight-based distribution, and molar-based information can be obtained only indirectly in combination with other means. Recently, labeling techniques with a fluorophore have been introduced to the structural analysis of the fractionated starch components amylose and amylopectin (Hanashiro & Takeda, 1998; Hanashiro, Takawa, Shibahara, Iwata, & Takeda, 2002). By this technique, direct measurement of molar-based as well as weight-based distributions of the molecules can be achieved.

According to the reports mentioned above, combining the procedure of amylose determination by HPSEC of debranched starch with the fluorescent-labeling technique and the detection of refractive index and fluorescent responses was thought to be useful for simultaneous determination of amylose content and unit chain distribution of amylopectin without starch fractionation. This paper describes the use of this proposed method for analyzing the starch samples isolated from four cassava varieties. The amylose contents obtained by this technique were compared with those determined by amperometric titration, whereas the unit chain distributions were compared with those analyzed on the fractionated amylopectins from corresponding cassava starches.

2. Materials and methods

2.1. Materials

Starch samples used in this study were isolated from 12-month roots of four cassava varieties: M-hanatee, F-rayong2, F-18 and MF-6. All chemicals used in this experiment were analytical grade.

2.2. Cassava amylopectin preparation

Amylopectin was prepared according to a procedure described by Takeda, Hizukuri, and Juliano (1987a) with some modifications. Starch (20 mg dry weight) was dissolved in 600 μl of 100% dimethyl sulfoxide (DMSO) by heating in a water bath for 30 min with stirring by Vortex mixer every 5 min. Dissolved starch was precipitated with absolute ethanol (600 μl), stored in ice box for 2 h and centrifuged at 3000 rpm, 4 °C for 10 min. The precipitate was redissolved and precipitated. It was then dispersed in water (3400 μl) at 70–80 °C, and a mixture of 1-butanol (200 μl) and 3-methyl-1-butanol (200 μl) was added. The dispersion was stirred and boiled under reflux for 3 h, cooled to 50 °C and kept in a Styrofoam box overnight at room temperature and then in cold room for 24 h. The supernatant separated by centrifugation (9000 rpm, 4 °C, 10 min) was precipitated with 3-fold of ethanol and stored in ice box for 2 h. The precipitate (amylopectin fraction) was collected by centrifugation (9000 rpm, 4 °C, 10 min) and washed with cold ethanol.

2.3. Unit chain distribution of cassava amylopectin

Cassava amylopectins (4 mg) from Section 2.2 were debranched with *Pseudomonas* isoamylase (Hayashibara Biochemical Lab., Inc., Okayama, Japan) and then labeled with a fluorescent reagent (2-Aminopyridine; 2-AP) (Hanashiro et al., 2002). The unit chain distribution profile of amylopectin was then determined by using high-performance size-exclusion chromatography with columns of Shodex OHpak SB-803HQ and SB-802.5HQ×2 (8×300 mm, Showa Denko, Tokyo, Japan) connected in series. The fluorescent response (F) and refractive index response (RI) were detected by a fluorescent detector (FS-8010, Tosoh) and a refractive index detector (ERC-7512, Erma, Inc., Tokyo, Japan), respectively. Aqueous DMSO (50%) containing 50 mM NaCl was used as eluent with flow-rate of 0.25 ml/min. Column temperature was kept at 50 °C. The percent fraction and unit chain length of A, B and B2+B3 chains, as well as the average chain length of amylopectin, were determined according to a method described by Hanashiro et al. (2002).

2.4. Simultaneous determination of amylose content and amylopectin structure

Starch was defatted by dissolution in DMSO and precipitation with ethanol. The defatted starch (4 mg) was dissolved in water (950 μl) with heating and 50 μl of 10 mM acetate buffer (pH 3.5) was added. Debranching of starch by isoamylase (0.03 U/mg starch) was carried out at 45 °C for 12 h. The starch isoamylolyzate obtained was dried by using centrifugal evaporator for 3–4 h. After re-dissolution in DMSO, the starch isoamylolyzate was labeled with 2-AP and its unit chain distribution profile was determined by the procedure described for debranched amylopectin.

2.5. Amylose content determination

Amylose content was determined by the following three methods: (1) by dividing the RI response of amylose fraction by the total RI response of debranched starch; (2) by subtracting the RI response of amylopectin from total RI response of debranched starch and then dividing by total RI response of debranched starch; (3) by the amperometric titration method (Larson, Gilles, & Jenness, 1953) with some modifications (Takeda et al., 1987a; Takeda, Hizukuri, Takeda & Suzuki, 1987b). Overestimation of amylose content by interaction of long unit chains of amylopectin with iodine was corrected by subtracting the iodine affinity of amylopectin from that of the defatted starch as in the

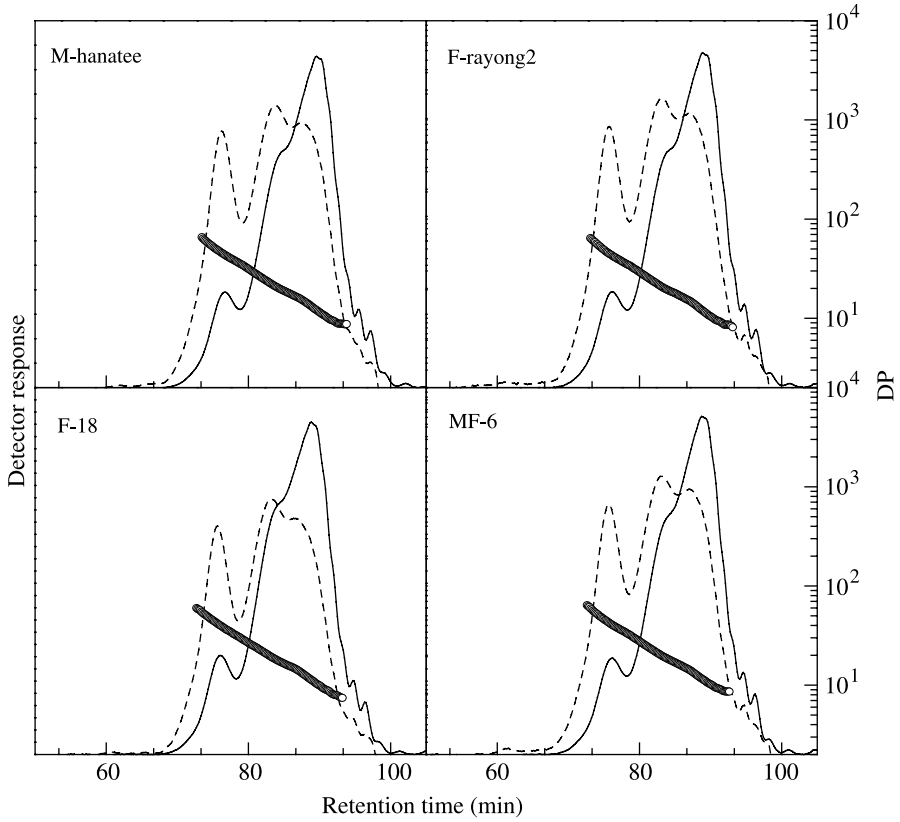

Fig. 1. Chromatograms of fluorescent-labeled unit chain of four cassava amylopectins. (- - -) Refractive index response, RI; (—) fluorescent response, F; (●) degree of polymerization.

equation:

$$(IA_{\text{defatted starch}} - IA_{\text{amylopectin}})/(IA_{\text{amylose}} - IA_{\text{amylopectin}}) \times 100.$$

3. Results and discussion

3.1. Unit chain distribution of amylopectin

The unit chain distributions of amylopectins from four varieties of cassava are shown in Fig. 1. All cassava amylopectins displayed two distinct peaks at DP 40–44 and the second peak at DP 11–12 with a shoulder at DP 17–19. The results were comparable to those reported by Asaoka, Blanshard, and Rickard (1991) (DP 37–48, 11–15 and 17–22), Hizukuri (1986) (DP 38, 18 and 11), and Thitipraphunkul, Uttapap, Piyachomkwan, and Takeda (2003) (DP 36, 16 and 10). An interesting point from our results was that extra-long chains (0.24–0.50%) were found in amylopectins of cassava starches. Extra-long chains existing in amylopectin of cassava starch had never been reported previously. The number-average chain length (CL) of cassava amylopectins was 18–20. These CL values were close to those (21 and 20) determined by chromatographic method (Suzuki, Takeda, & Hizukuri, 1985) and HPSEC/fluorescent-labeling method (Thitipraphunkul et al., 2003), but lower than those (28 and 25) determined by using HPAEC-ENZ-PAD chromatography (Jane et al., 1999) and colorimetric method with isoamylolysis (Gunaratne & Hoover, 2002), respectively.

The area under the distribution profile of amylopectin was divided into A-, B1- and B2 + B3-chain fractions (vertical solid and dotted lines for mole and mass fractions, respectively) as shown in Fig. 2. According to Hizukuri's model (1986), the molecular species that is represented by the first peak area (retention time = 68–78 min) in the profile of amylopectin fraction was identified as long B-chains (B2 + B3), where the

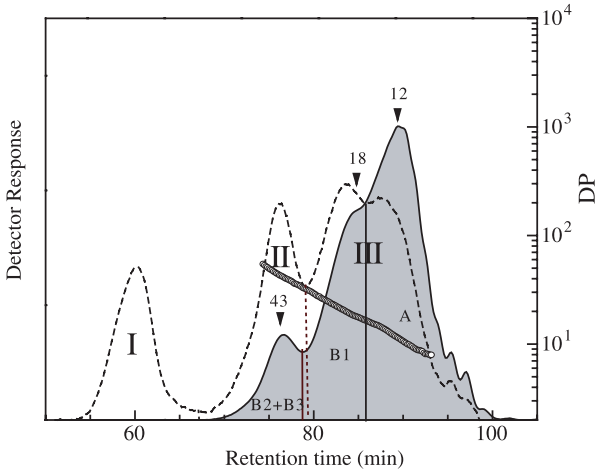

Fig. 2. Chromatogram of fluorescent-labeled isoamylolyzate of M-hanatee starch. (- - -) Refractive index response, RI; (—) fluorescent response, F; (●) degree of polymerization. Arrows indicate DP values.

Table 1
Properties of unit chains of four cassava amylopectins determined from chromatograms of fractionated amylopectins

Distribution of unit chain	Sample			
	M-hanatee	F-rayong2	F-18	MF-6
Unit chain (% mole)				
A	60.60±0.07	60.12±0.07	59.30±0.33	60.33±1.20
B1	28.30±0.15	28.87±0.22	29.36±0.21	28.75±1.23
B2+B3	11.10±0.08	11.01±0.15	11.34±0.12	10.92±0.02
A+B1/B2+B3	8.01±0.07	8.08±0.13	7.82±0.10	8.16±0.02
Unit chain (% weight)				
A	35.19±0.10	36.69±0.39	33.33±1.06	35.14±0.33
B1	34.98±0.13	33.70±0.39	37.17±0.84	35.24±0.22
B2+B3	29.37±0.53	29.11±0.23	29.27±0.16	29.10±0.02
A+B1/B2+B3	2.39±0.05	2.42±0.02	2.41±0.02	2.42±0.01
Extra long chain (%)	0.46±0.30	0.50±0.23	0.24±0.06	0.51±0.10
Average chain length (CL)	19.39±0.01	19.64±0.61	17.56±0.42	18.01±0.69

All values are the means of duplicate measurements.

second (retention time = 78–86 min) and third peak area (retention time = 86–100 min) represented B1 and A chains, respectively. The resulting percent fractions of A, B1 and B2+B3 chains of amylopectins based on both molar- and weight-based distributions are summarized in Table 1. Mole percent fraction of A, B1 and B2+B3 chains was 59–61, 28–29 and 11%, and molar ratio of A+B1 and B2+B3 chains was in a range of 7.8–8.1. Based on the mass fraction, however, A, B1 and B2+B3 chains presented in approximately equal amounts (33–37, 34–37 and 29%) and the weight ratio of A+B1/B2+B3 was 2.4. These were close to those reported as 2.45 by Hizukuri (1986) and 2.6 by Ong, Jumel, Tokarczuk, Blanshard, and Harding (1994). The results obtained from this section were used as references for comparison with those from the starch isoamylolyzate.

3.2. Simultaneous determination of amylose content & unit chain distribution of amylopectin

3.2.1. Chromatograms of starch isoamylolyzate

Simultaneous determination of amylose content and unit chain distribution of amylopectin was done by debranching the starch, labeling the isoamylolyzate with fluorophore and separating the labeled products with high-performance size-exclusion chromatography. Both refractive index and fluorescent responses of separated species were recorded. Fig. 2 demonstrates a sample of a chromatogram using starch from the M-hanatee cassava variety as a representative.

For the chromatogram of RI response (dotted line), it was clearly seen that there were two peaks separated completely. The products of starch digestion by isoamylase should comprise two main fractions: one containing chains with a high degree of polymerization (DP) from the amylose, and a

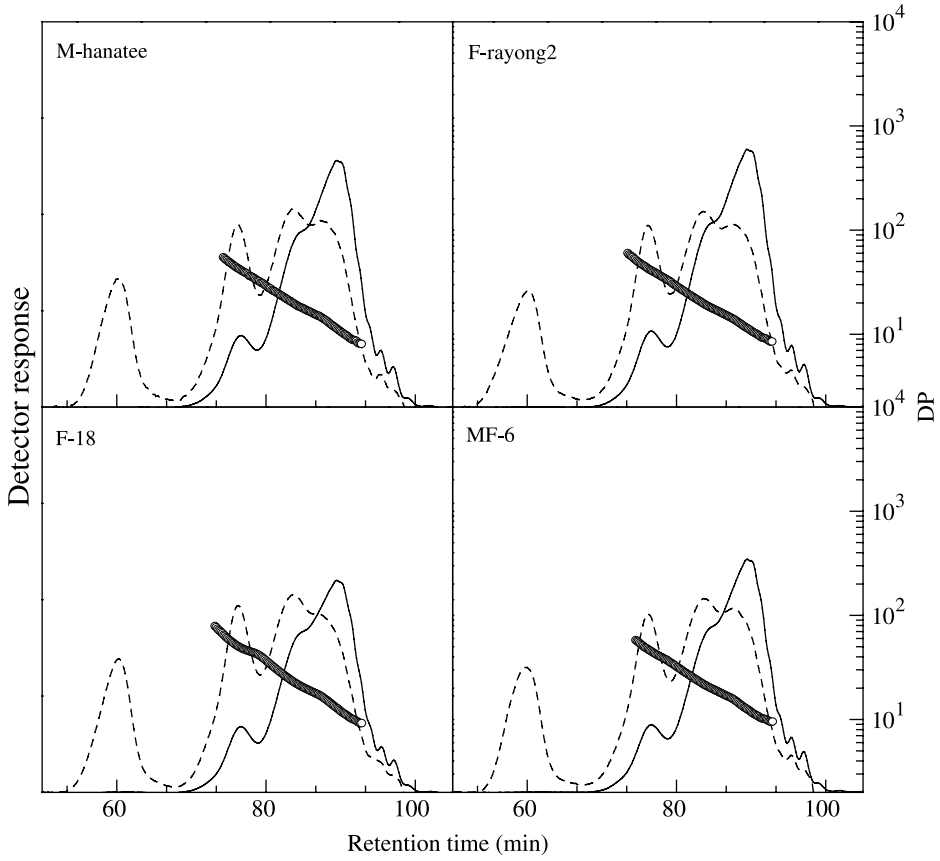

Fig. 3. Chromatograms of fluorescent-labeled isoamylolyzate of four cassava starches. (- - -) Refractive index response, RI; (—) fluorescent response, F; (●) degree of polymerization.

Table 2
Amylose content of four cassava amylopectins

Sample	Amylose content (%)		
	$Amylose_{starch\ isoamylolyzate}$	$Amylose_{starch\ \&\ amylopectin\ isoamylolyzate}$	$Amylose_{amperometric}$
M-hanatee	17.14±0.23	17.33±0.19 (−0.19)[a]	17.80±0.06 (−0.66)[b]
F-rayong2	17.50±0.59	17.24±1.03 (0.26)	18.65±0.07 (−1.15)
F-18	17.78±0.28	18.03±0.02 (−0.25)	19.11±0.06 (−1.33)
MF-6	17.74±0.44	18.81±1.34 (−1.07)	17.64±0.03 (0.10)

All values are the means of duplicate measurements.
[a] Values in parentheses are the differences between $amylose_{starch\ isoamylolyzate}$ and $amylose_{starch\ \&\ amylopectin\ isoamylolyzate}$.
[b] Values in parentheses are the differences between $amylose_{starch\ isoamylolyzate}$ and $amylose_{amperometric}$.

second fraction consisting of chains of less than ≈100 glucose units derived from amylopectin. That is, the first peak in the chromatogram would be a peak of the amylose fraction and the second peak would be of the amylopectin fraction. The completely separated peaks indicated that the three columns connected in series (Shodex OHpak SB-803HQ and SB-802.5HQ×2), which have been used for separation of unit chains of amylopectin, can be efficiently applied for separation of digested products of amylose from the digested products of amylopectin.

For the chromatogram of fluorescent response (solid line), only a peak of amylopectin fraction was found. Investigation of molecular size of amyloses from starches of these four cassava varieties revealed that DP of amyloses was approximately at 4000, and nearly half of amylose molecules were linear chains without branch (data will be reported elsewhere). Regarding the branched amylose fraction, when debranched, chains having more than 500 glucose units would be obtained. By contrast, the average chain length of cassava amylopectins as mentioned in Section 3.1 was merely between 18 and 20. In addition, the amounts of amylopectin in cassava starch were much higher than those of the amylose (about 80:20). Therefore, the numbers of reducing end of amylose fraction were much less than those of amylopectin fraction. As a consequence, the fluorescent response of amylose fraction was relatively much less when compared to that of the amylopectin fraction (the fluorescent detector recorded the fluorescent activities of fluorophore labeled at the reducing end of each unit chain). In this experiment, the response of the amylose fraction was too small to be detected.

3.2.2. Determination of amylose content

Only RI response reflecting a mass of each fraction was used for amylose content calculation. Fractions I, II and III of RI response (Fig. 2) were regarded as amylose, long chains of amylopectin and short chains of amylopectin, respectively. Each fraction was divided at the lowest point of RI response. Area of fraction I divided by sum of fraction I, II and III area corresponded to the content of amylose (termed as $amylose_{starch\ isoamylolyzate}$).

Distribution profiles of isoamylolyzates of starches from four varieties of cassava are shown in Fig. 3. The amylose content was calculated from the chromatograms described previously, and the results are summarized in Table 2. The amylose contents of all varieties were between 17.1 and 17.8%. The values obtained for each starch sample were highly reproducible for duplicate analyses with standard deviations less than 0.6%. This indicated that the method of HPSEC of labeled starch isoamylolyzate is applicable for measuring small differences in starches of similar amylose content. The amylose content of starches from these four varieties was in the same range as those of cassava starch measured by gel permeation chromatography (16–20%) (Asaoka et al., 1991) and by the method of amperometric titration (18%) (Thitipraphunkul et al., 2003).

It has been reported that amylose consists of two fractions: linear and branched fractions (Takeda et al., 1987b). The amounts of branched amylose in canna, mung bean and KU50 cassava starches were 13–16, 16, and 34%, respectively (Thitipraphunkul et al., 2003). During isoamylolysis, the branched molecules were hydrolyzed and the very short branch chains, if they existed, might be fractionated in the amylopectin fraction. On the other hand, extra-long unit chains of amylopectin, if they existed, might be fractionated in the amylose fraction. This may result in some errors of amylose determination by the HPSEC/fluorescent labeling technique of

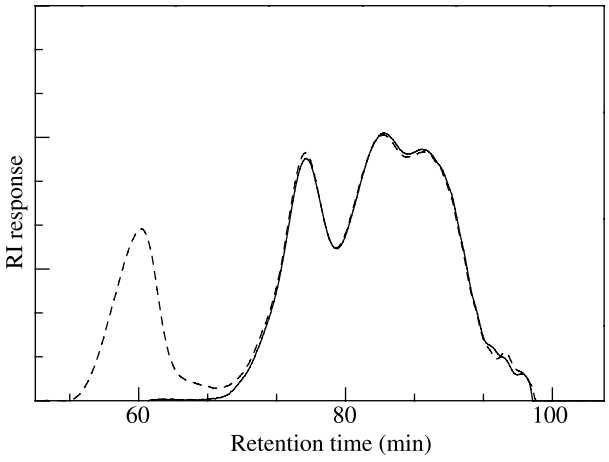

Fig. 4. Chromatograms of isoamylolyzate of M-hanatee starch and fractionated amylopectin. (- - -) RI response of starch isoamylolyzate; (—) RI response of fractionated amylopectin.

Table 3
Properties of unit chains of four cassava amylopectins determined from chromatograms of starch isoamylolyzates

Unit chain	Sample			
	M-hanatee	F-rayong2	F-18	MF-6
Unit chain (% mole)				
A	61.80±0.20 (−1.20)	62.81±0.06 (−2.69)	60.94±0.18 (−1.64)	62.61±0.14 (−2.27)
B1	27.14±0.40 (1.16)	26.22±0.16 (2.65)	27.61±0.03 (1.75)	26.27±0.18 (2.43)
B2+B3	11.05±0.19 (0.05)	10.97±0.21 (0.04)	11.45±0.20 (−0.11)	11.11±0.04 (−0.15)
A+B1/B2+B3	8.05±0.16 (−0.04)	8.12±0.18 (−0.04)	7.74±0.16 (0.08)	8.00±0.03 (0.12)
Unit chain (% weight)				
A	35.52±0.35 (−0.33)	36.55±1.07 (0.14)	29.72±0.29 (3.61)	35.87±0.05 (−0.49)
B1	33.79±0.24 (1.19)	33.46±1.24 (0.24)	39.32±0.02 (−2.15)	34.11±0.27 (0.98)
B2+B3	29.69±0.60 (−0.32)	29.99±0.16 (−0.88)	30.96±0.31 (−1.69)	30.02±0.22 (−0.93)
A+B1/B2+B3	2.37±0.07 (0.02)	2.33±0.02 (0.09)	2.24±0.03 (0.17)	2.33±0.02 (0.09)
Average chain length (CL)	18.63±0.41 (0.76)	18.08±0.96 (1.56)	19.87±0.25 (−2.31)	18.79±0.20 (0.37)

All values are the means of duplicate measurements. Values in parentheses are the differences between the values obtained from isoamylolyzates of amylopectin and starch.

starch isoamylolyzate. To assess the reliability of the amylose determination by this method, the values obtained were compared to those determined by amperometric titration (termed as $\text{amylose}_{\text{amperometric}}$) and by subtracting the response of debranched amylopectin from the response of debranched starch (termed as $\text{amylose}_{\text{starch \& amylopectin isoamylolyzate}}$). To determine the $\text{amylose}_{\text{starch \& amylopectin isoamylolyzate}}$, RI response profile of total starch isoamylolyzate was overlaid with the RI response profile of isoamylolyzate of counterpart amylopectin. Overlaying of RI response of amylopectin on RI response of debranched starch from the same starch sample (Fig. 4) revealed that the response line of amylopectin (solid line) fitted well to the response line of debranched starch (dotted line). The data summarized in Table 2 shows that the values of the $\text{amylose}_{\text{starch isoamylolyzate}}$ were about 1% less than those of the $\text{amylose}_{\text{amperometric}}$ and were very close to (about ±0.3%) those of the $\text{amylose}_{\text{starch \& amylopectin isoamylolyzate.}}$ The slight difference was thought to arise at the turning point from amylose peak to amylopectin peak, and was supposed to be due to the extra-long chains of cassava amylopectins.

From the results obtained, it can be concluded that this technique is highly reproducible and appropriate for measurement of the amylose content in starches containing amylopectin with low/no extra-long chains. Generally, the extra-long chains have been found in cereal amylopectin such as rice (6–20%) (Hizukuri, Takeda, Maruta, & Juliano, 1989), wheat (4–6%) (Shibanuma, Takeda, & Hizukuri, 1994), and maize (10–11%) (Takeda, Shitaozono, & Hizukuri, 1988), but small amounts have been reported in sago (0.4–2.6%) (Takeda, Takeda, Suzuki, & Hizukuri, 1989) and potato (0.8%) (Suzuki, Shibanuma, Takeda, Abe, & Hizukuri, 1994). As stated by Batey et al. (1996), precipitation of the amylose in isoamylolyzate may occur if the starches contain amylose content higher than 35%. For our method, however, precipitation is believed not to occur even with the pure amylose solution, since there was no such problem in chromatography of amylose solution prepared by the same procedure as this report (Hanashiro et al., 1998).

3.2.3. Unit chain distribution of amylopectin

By this technique, not only the amylose content but also unit chain distribution of amylopectin could be obtained. The unit chain distribution profiles of amylopectins from four varieties of cassava obtained by starch debranching and labeling/HPSEC method are illustrated in Fig. 3. It is clearly seen that these profiles are very similar to those of the fractionated amylopectins (Fig. 1), except that the extra-long chain fraction could not be seen in the starch isoamylolyzate distribution. This is due to overlapping between peak area of amylose and amylopectin at around retention time of 58–70 min. As a consequence, the extra-long chain fraction could not be identified by this procedure.

The molar- and weight-based unit-chain fraction and ratio of (A+B)/(B2+B3) calculated from the distribution profiles of starch isoamylolyzates in Fig. 3 are summarized in Table 3. The mole fractions (%) of A, B1 and B2+B3 were 61–63, 26–28 and 11, respectively. Their molar ratios of A+B1 and B2+B3 were 7.7–8.1. These values were comparable to the values obtained from fractionated amylopectins (Table 1: mole fractions of A, B1 and B2+B3 were 59–61, 28–29 and 11, respectively, and molar ratio of A+B1 and B2+B3 was 7.8–8.1). However, the differences in mole fractions of A and B1 chains (about 1–2.5%) were higher than those of B2+B3 chains (about 0.04–0.15%). This was due to the inaccuracy of judging the separated line (Fig. 2) between the peaks of A and B1 chains. Similarly, the data based on weight distribution was comparable to that from the fractionated amylopectins. The mass fractions of A, B1 and B2+B3 from chromatograms of starch isoamylolyzates were 30–37, 33–39 and 30–31%, respectively, whereas those from fractionated amylopectins were 33–37, 34–37 and 29%, respectively.

4. Conclusion

The fluorescent labeling of debranched starch/HPSEC method was proved to be efficient for simultaneous determination of unit chain distribution of amylopectin as well as amylose content of cassava starches. The amylose contents of

cassava starch samples determined by this technique were comparable to those determined by amperometric titration method and by overlaying the amylopectin profile on the profile of starch isoamylolyzate. The distribution profile of amylopectin and percent fraction of unit chains were also comparable to those obtained from the fractionated amylopectins. However, the limitation of this method was that the extra-long chains, if they existed, would be hindered by a peak of amylose. Thus, this method may not be suitable for cereal starch analyses since these starches usually contain some extra-long chains. In conclusion, this method is at least applicable for analysis of tuber/root starch containing low or medium amylose. Further investigation of application of this method for analyses of high-amylose starches should be carried out.

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